Attorney Docket No.: 20488/26-DIV

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants:	Demuth et al.	Art Unit:	1654
Serial No.:	09/682,968	Confirmation No.:	3916
Filed:	November 2, 2001	Examiner:	Michael V. Meller
For:	METHOD FOR RAISING THE BLOOD GLUCOSE LEVEL IN MAMMALS		

CERTIFICATE OF MAILING

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By: Sandra Wittrup
Sandra J. Wittrup

November 5, 2003
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ATTENTION: Board of Patent Appeals and Interferences

APPELLANTS' BRIEF (37 C.F.R. 1.192)

- This brief is in furtherance of the Notice of Appeal, filed in this case on July 25, 2003.
- The fees required under § 1.17(c), and a petition for extension of time for filing this brief and fees therefore, are provided within the accompanying TRANSMITTAL OF APPEAL BRIEF. If any fee is missing or insufficient please charge Deposit Account 50-0369. If the petition for extension of time for filing this brief is missing, please consider this paper such a petition.
- This brief is transmitted in triplicate. (37 C.F.R. 1.192(a))
- This brief contains the following items under the following headings, and in the order set forth below (37 C.F.R. 1.192(c)).

- TABLE OF CONTENTS

The final page of the arguments bears the practitioner's signature

(i) TABLE OF AUTHORITIES

FEDERAL CASES

FEDERAL STATUTES

OTHER AUTHORITIES

I. REAL PARTIES IN INTEREST (37 C.F.R. 1.192(c)(1))

The real party in interest in this appeal is the following party: probiodrug AG., as the assignee of record in the parent application of which the present application is a division thereof.

II. RELATED APPEALS AND INTERFERENCES (37 C.F.R. 1.192(c)(2))

With respect to other appeals or interferences that will directly affect, or be directly affected by, or have a bearing on the Board's decision in the pending appeal there are no such appeals or interferences.

III. STATUS OF CLAIMS (37 C.F.R. 1.192(c)(3))

A. TOTAL NUMBER OF CLAIMS IN APPLICATION

Claims in the application are: 1-5.

B. STATUS OF ALL THE CLAIMS IN APPLICATION

1. Claims canceled: 6-11
2. Claims withdrawn from consideration but not canceled: NONE
3. Claims pending: 1-5
4. Claims allowed: NONE
5. Claims rejected: 1-5

C. CLAIMS ON APPEAL

The claims on appeal are: 1-5.

IV. STATUS OF AMENDMENTS (37 C.F.R. 1.192(c)(4))

No amendment to the claims 1-5 has been filed in response to the Final Rejection, therefore no amendment has been entered after the Final Rejection.

V. SUMMARY OF INVENTION (37 C.F.R. 1.192(c)(5))

The present invention relates to the use and administration of effectors of Dipeptidyl Peptidase ("DP IV") and DP IV-analogous enzyme activity in and to mammals for the prevention or alleviation of pathological metabolic anomalies. Such an anomaly may be, for example, acute or chronic hypoglycemia where rapid mobilization of carbohydrate reserves of the liver is necessary.

The invention also relates to use and a method for raising the blood sugar level in the serum of a mammalian organism above the glucose concentration characteristic of hypoglycemia. According to the invention, a therapeutically effective amount of an effector of DP IV and DP IV-analogous enzyme activity is administered to a mammalian organism.

The present invention advantageously imposes a low therapeutic burden on the subject organism, since only small doses of external hormone, if any, need to be administered. According to the invention, glucagon degradation is decelerated or completely stopped by the use of effectors of DP IV and DP IV-analogous enzyme activity.

The effectors of DP IV and DP IV-analogous enzymes administered according to the invention may be used in pharmaceutically administrable formulations complexed as inhibitors, substrates, pseudosubstrates, inhibitors of DP IV expression, binding proteins or antibodies to those enzyme proteins or combinations of those different substances that reduce the DP IV or DP IV-analogous protein concentration in the mammalian organism. Effectors used according to the invention can comprise, for example, DP IV inhibitors, such as the dipeptide derivatives and dipeptide mimetics alanyl-pyrolidide, isoleucyl-thiazolidide, and the pseudosubstrate N-valyl-prolyl, O-benzoyl hydroxylamine or salts thereof, especially fumarates thereof.

VI. ISSUES (37 C.F.R. 1.192(c)(6))

Issue 1: Whether the phrase "an effector for reducing the enzymatic activity of DPIV" within claim 1 (and dependent claim 2—5) is enabled under the requirements of 35 U.S.C. § 112, first paragraph.

Issue 2: Whether the term "effectors" within claims 1-5 is distinctly claimed under the requirements of 35 U.S.C. § 112, second paragraph.

Issue 3: Whether claims 1-5 constitute double patenting over claims 1-4 of U. S. Patent No. 6,319,893.

VII. GROUPING OF CLAIMS (37 C.F.R. 1.192(c)(7))

Issue 1: Claim 3 is grouped separately from Claims 1 through 5 as to whether the phrase "an effector for reducing the enzymatic activity of DPIV" within Claim 1 (and dependent claim 2—5) is enabled under the requirements of 35 U.S.C. § 112, first paragraph.

Issue 2: Claim 3 is grouped separately from Claims 1 through 5 as to whether the term "effectors" within Claims 1-5 is distinctly claimed under the requirements of 35 U.S.C. § 112, second paragraph.

VIII. ARGUMENT (37 C.F.R. 1.192(c)(8)(iv)):

ISSUE 1: WHETHER THE PHRASE "AN EFFECTOR FOR REDUCING THE ENZYMATIC ACTIVITY OF DPIV" WITHIN CLAIMS 1 (AND DEPENDENT CLAIM 2—5) IS ENABLED UNDER THE REQUIREMENTS OF 35 U.S.C. § 112, FIRST PARAGRAPH.

A: The Rejection -- In the Office Action dated January 27, 2003, the Examiner rejected Claims 1-11 under 35 U.S.C. § 112. The rejection was based on the phrase in independent Claim 1: "an effector for reducing the enzymatic activity of DPIV." In the interest of completeness, Applicant assumes that this includes DPIV and DPIV analogous enzymes.

Claim 1 contains this language and Claims 2 through 5 are dependent thereon.

> 1. A method of raising the blood sugar level in a mammal having hypoglycemia by reducing degradation of glucagon, said method comprising administering to said mammal a therapeutically effective amount of an effector for reducing enzymatic activity of dipeptidyl peptidase (DP IV) and DP IV-analogous enzymes.

The Examiner stated that the specification was enabling for DPIV administration:

> ". . . [W]hile being ***enabling*** *for a method of administering to a mammal a therapeutically effective amount of an inhibitor of DPIV and physiologically acceptable adjuvants and/or excipients for reducing in said mammal activity of endogenous DPIV*, does not reasonably provide enablement for administering any and all effectors for reducing enzymatic activity of DPIV and DPIV analogous enzymes. (emphasis added) Office Action of January 27, 2003, at 2-3.

In making this rejection the Examiner has acknowledged enablement in the claimed method of administering a drug. Furthermore the Examiner has acknowledged enablement of the end point -- reducing in said mammal activity of endogenous DPIV. The Examiner's rejection appears to focus in the Examiner's view of enablement as to "any and all effectors." The Examiner is not convinced of enablement.

B. Not required to convince: It is respectfully noted that Appellants are not required to convince. In the absence of evidence or apparent reason why the invention does not perform as claimed, the allegation of enablement in the Specification must be

accepted as correct. See, In re Bundy, 209 U.S.P.Q. 48 (CCPA 1981) (addressing utility).

C. Efficacy need not be proven: The Advisory Action of June 3, 2003, at Continuation of 5, indications that the rejection is maintained , in part, because "[t]he art of biotechnology is has a very high level of unpredictability" and continues to fault the application for lack of proof of that the claimed result will arise. This is an improper basis for rejection. "

> There is nothing in the patent statute or any other statutes called to our attention which gives the Patent Office the right or duty to require an applicant to prove that compounds or other materials which he is claiming, and which he had stated are useful for 'pharmaceutical applications' are safe, effective, and reliable for humans. See Carter-Wallace, Inc. v. Riverton Labs., 167 U.S.P.Q. 656, 660-61 (2d. Cir. 1970)

Insofar as the rejection is based on the absence of proof of effective the rejection is speculative and improper.

D. Non-inhibitory effector compounds enabled: The Examiner finds enablement only for inhibitory compounds. Office Action of January 27, 2002, last sentence at 2 continuing to 3. This position is traversed. Contrary to the Examiner's position, Appellant's specification in paragraph 47 identifies other effector compounds capable of raising the blood sugar level in a mammal having hypoglycemia by reducing degradation of glucagons. Two disclosed compounds are substrates and pseudosubstrates. Substrates and pseudosubstrates raise the blood sugar levels in hypoglycemia by reducing degradation of glucagon – not by inhibition of DPIV but by increasing the targets upon which DPIV can act. It is submitted that recitation of "substrates and pseudosubstrates" would clearly be understood by one skilled in the art – a prescribing physician -- to influence the enzymatic activity of DPIV by mechanisms other than inhibition. Similarly, Claim 3 as filed specifies -- along with DP IV enzyme inhibitors – classes of non-inhibitory effectors. These include substrates of DP IV and pseudo-substrates of DP IV, as well as inhibitors of DP IV expression, proteins that bind DP IV and antibodies to DP IV.

Instructive guidance in determining the level of ordinary skill has been provided by the Federal Circuit as follows:

> "The person of ordinary skill is a hypothetical person who is presumed to be aware of all the pertinent prior art. The actual inventor's skill is not determinative. Factors that may be considered in determining level of skill include; type of problems encountered in art; prior art solutions to those problems. Rapidity with which innovations are made; sophistication of the technology; and educational level of active workers in the field." Custom Accessories Inc. V. Jeffrey-Allan Indus., 807 F.2d 955, 1 U.S.P.Q. 2d 1196, 1201 (Fed. Cir. 1986).

Substrates and pseudosubstrates are explicitly identified as effectors within the specification. In physiological concentrations these are not inhibitors of DPIV. Inhibitors of DP IV expression are not inhibitors of DPIV, proteins that bind DP IV and antibodies to DP IV are not necessarily inhibitors of DPIV activity. It is settled law that a specification, drawn to one of ordinary skill in the art, need only describe enough information to allow one of ordinary skill in the art to make the invention work. Ex parte Naujoks, 17 U.S.P.Q.2d 1537, 1540 (PBAI 1989). It is submitted that the existence and identity of substrates and pseudosubstrates are known to those of skill in the art, and having been cited are enabled. The Examiner has advanced no reasoning that contests the availability of such substrates, pseudosubstrates, inhibitors of DP IV expression, proteins that bind DP IV and antibodies to DP IV.

Appellants respectfully submit that the breadth of the Claims 1 is clearly enabled by the instant specification, and Claims 2 through 5 dependent thereon.

E. Claim 3 Specifies Effectors:

> Claim 3. The method of claim 1, wherein the effector is selected from the group consisting of DP IV enzyme inhibitors, substrates of DP IV, pseudo-substrates of DP IV, inhibitors of DP IV expression, proteins that bind DP IV or antibodies to DP IV and combinations thereof.

The rejection is based on enablement, *vel non*, of administering any and all effectors. Claim 3 specifies DP IV enzyme inhibitors, substrates of DP IV, pseudo-substrates of DP IV, inhibitors of DP IV expression, proteins that bind DP IV or antibodies to DP IV and combinations thereof. This litany is specific and in keeping with the requirement of 35 U.S.C. § 112, first paragraph.

Appellants respectfully submit that the breadth of the Claim 3 is clearly enabled by the instant specification. Appellant respectfully requests that this rejection be withdrawn.

ISSUE 2: ISSUE 2: WHETHER THE TERM "EFFECTORS" WITHIN CLAIMS 1-5 IS DISTINCTLY CLAIMED UNDER THE REQUIREMENTS OF 35 U.S.C. § 112, SECOND PARAGRAPH.

A. The rejection: In the Office Action dated January 27, 2003, the Examiner rejected claims 1-5 and 7-11 under 35 U.S.C. 112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which Appellant regard as its invention. As noted above claims 7-11 have been cancelled. Remaining claims 1-5 have been rejected for failing to particularly point out and distinctly claim the subject matter of the invention due to use of the word "effector." Specifically, the Examiner objected to the phrase "an effector for reducing enzymatic activity of DPIV and DPIV analogous enzymes" as being confusing and therefore failing to clearly point out Appellants' invention. Appellant respectfully traverses this rejection.

B. Effector is Definite: The record includes clarifying usage of the word "effector" as found in Molecular Cell Biology, Second Edition, Ed. Darnell et al (Scientific American Books, 1990). (attached here as Appendix A) Molecular Cell Biology is a textbook in the pharmaceutical art written and edited by three well known and respected Nobel prize laureates. This chapter was submitted by facsimile on April 9, 2003, and resubmitted by Supplemental Amendment on June 26, 2003. Attention is drawn to page 63, upper right hand column: "Molecules that bind to enzymes and increase or decrease their activities are called *effectors*." (emphasis in original).

By italicization of "effector," Molecular Cell Biology defines "effector" as a definite term in the art. The term effector means a molecule that binds to enzymes and either increases or decreases the enzymatic activity. As such, the term effector meets the requirements of 35 U.S.C. 112, second paragraph.

The Examiner has failed to set forth a *prima facie* case of indefiniteness as required by statute. The Board of Appeals has specified the basis for a proper rejection under 35 U.S.C. 112 as follows:

> "In rejecting a claim under the second paragraph of 35 U.S.C. 112, it is incumbent on the examiner to establish that one of ordinary skill in the pertinent art, when reading the claims in light of the supporting specification, would not have been able to ascertain with a reasonable degree of precision and particularity

the particular area set out and circumscribed by the claims" Ex parte Wu, 10 U.S.P.Q. 2d 2031 (emphasis added).

Appellant respectfully requests that this rejection be overturned as to all pending claims, Claims 1 through 5.

C. Claim 3 Specifies Effectors:

Claim 3. The method of claim 1, wherein the effector is selected from the group consisting of DP IV enzyme inhibitors, substrates of DP IV, pseudo-substrates of DP IV, inhibitors of DP IV expression, proteins that bind DP IV or antibodies to DP IV and combinations thereof.

The rejection is based on the definiteness, *vel non*, of the term "effector." Claim 3 lists particular effectors: DP IV enzyme inhibitors, substrates of DP IV, pseudo-substrates of DP IV, inhibitors of DP IV expression, proteins that bind DP IV or antibodies to DP IV and combinations thereof. This litany defines effectors as one of six species. As such, Claim 3 meets the definiteness requirement of 35 U.S.C. § 112, second paragraph. Appellant respectfully requests that this rejection be withdrawn.

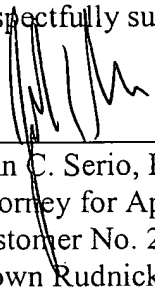
ISSUE 3: WHETHER CLAIMS 1-5 CONSTITUTE DOUBLE PATENTING OVER CLAIMS 1-4 OF U. S. PATENT NO. 6,319,893.

In the Office Action dated January 27, 2003, the Examiner has provisionally rejected claims 1-11 under the judicially created doctrine of double patenting over claims 1-4 of U.S. Patent No. 6,319,893. As noted above Appellant has cancelled claims 6-11 leaving only claims 1-5 at issue here. Appellant, upon notification of allowable subject matter, will execute an acceptable terminal disclaimer to overcome this rejection.

CONCLUSION

For the reasons advanced above, Appellant respectfully contends that each claim is patentable. Therefore, reversal of all rejections is earnestly solicited.

Respectfully submitted,



John C. Serio, Reg. No. 39,023
Attorney for Appellant
Customer No. 21710
Brown Rudnick Berlack Israels LLP
One Financial Center
Boston, MA 02111
Phone: 617-856-8238
Fax: 617-856-8201
e-mail: ip@brbilaw.com

Dated: November 5, 2003

IX. APPENDIX: CLAIMS INVOLVED IN THE APPEAL (37 C.F.R. 1.192(c)(9))

The text of the claims involved in the appeal is:

1. (PREVIOUSLY AMENDED) A method of raising the blood sugar level in a mammal having hypoglycemia by reducing degradation of glucagon, said method comprising administering to said mammal a therapeutically effective amount of an effector for reducing enzymatic activity of dipeptidyl peptidase (DP IV) and DP IV-analogous enzymes.

2. (ORIGINAL) The method of claim 1, wherein the blood sugar level in the serum of mammalian organisms is raised above the glucose concentration characteristic of hypoglycemia.

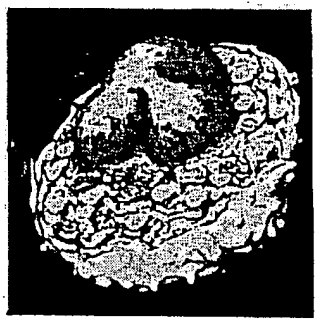
3. (PREVIOUSLY AMENDED) The method of claim 1, wherein the effector is selected from the group consisting of DP IV enzyme inhibitors, substrates of DP IV, pseudo-substrates of DP IV, inhibitors of DP IV expression, proteins that bind DP IV or antibodies to DP IV and combinations thereof.

4. (PREVIOUSLY AMENDED) The method of claim 1, wherein the effector for reducing enzymatic activity is employed together with glucagon or analogues thereof.

5. (PREVIOUSLY AMENDED) The method of claim 1, wherein the effector for reducing enzymatic activity is employed in combination with physiologically acceptable adjuvants and/or excipients.

Molecular Cell Biology

SECOND EDITION



JAMES DARNELL
Vincent Astor Professor
Rockefeller University

HARVEY LODISH
Member of the Whitehead Institute for Biomedical Research
Professor of Biology, Massachusetts Institute of Technology

DAVID BALTIMORE
President
Rockefeller University

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APPENDIX A

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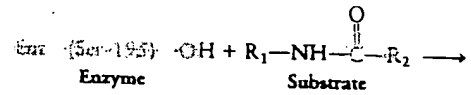
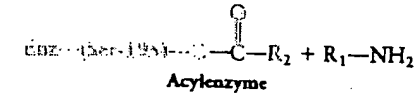
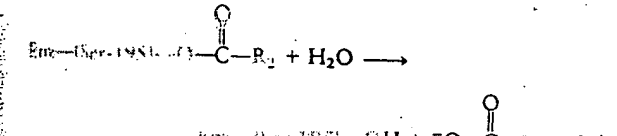
The hydrolysis reaction proceeds in two main steps. First, the peptide bond is broken and the carboxyl group is transferred to the hydroxyl residue of serine 195:





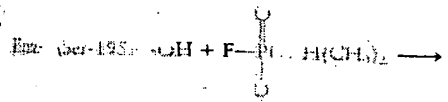
Second, this *acylenzyme* intermediate is hydrolyzed:

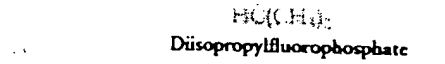


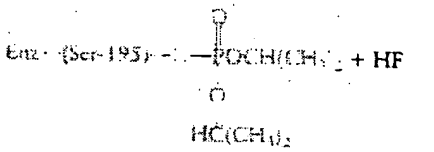
Note that the second step restores the enzyme to its original state.

Aspartate 102 and histidine 57 facilitate the acylation reaction by removing the proton from serine 195 and adding it to the nitrogen of the departing amino group (Figure 2-20). In a similar manner, aspartate 102 and histidine 57 facilitate the hydrolysis of the acylenzyme. These enzymatically catalyzed steps—transfer of a proton from the enzyme to the substrate, formation of a covalent acylserine intermediate, and hydrolysis of the acylenzyme—all drastically reduce the overall activation energy of the proteolysis reaction.

The hydroxyl group on serine 195 is unusually reactive. The concept of an "active" serine residue at the active site predated the determination of the crystal structure of chymotrypsin. It was already known, for example, that the compound diisopropylfluorophosphate is a potent inhibitor of chymotrypsin; it reacts only with the hydroxyl on serine 195 to form a stable covalent compound that irreversibly inactivates the enzyme:





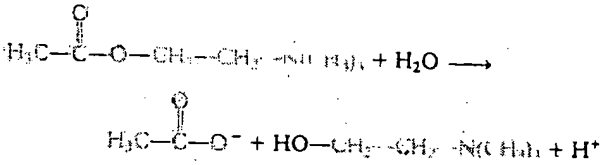


Trypsin and Chymotrypsin Have Different Substrate-binding Sites A comparison of trypsin and chymotrypsin will emphasize the nature of the specificity of enzymatically catalyzed reactions. About 40 percent of the amino acids in these two molecules are the same; in particular, the amino acid sequences in the vicinity of the key serine residue are identical:

195
—Gly—Asp—Ser—Gly—Gly—Pro

The three-dimensional structures and catalytic mechanisms of these two enzymes are also quite similar, indicating that they evolved from a common polypeptide. The major difference between trypsin and chymotrypsin is found in the side chains of the amino acids that line the substrate-binding site. The negatively charged amino acids in this area of the trypsin molecule facilitate the binding of only positively charged (lysine or arginine) residues, instead of hydrophobic ones.

Other Hydrolytic Enzymes Contain Active Serine Other, mostly unrelated, hydrolytic enzymes also contain an active serine residue that is essential for catalysis. For example, acetylcholinesterase catalyzes the hydrolysis of the neurotransmitter acetylcholine to acetate and choline:

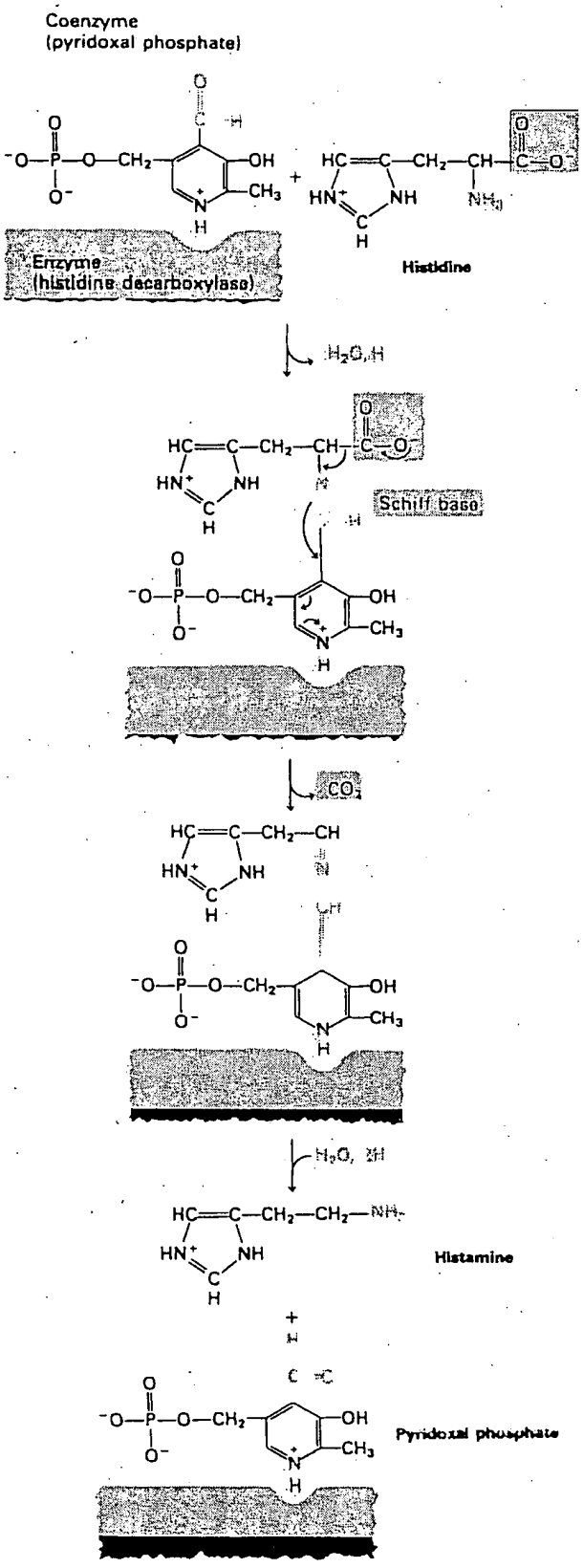


Diisopropylfluorophosphate is a potent, irreversible inhibitor of acetylcholinesterase as well as of chymotrypsin. The compound is lethal to animals because it blocks nerve transmission by causing a buildup of the transmitter substance. (The action of this transmitter is discussed in Chapter 20.)

Coenzymes Are Essential for Certain Enzymatically Catalyzed Reactions

Many enzymes contain a *coenzyme*—a tightly bound small molecule or prosthetic group essential to enzymatic activity. Vitamins required in trace amounts in the diet are often converted to coenzymes. Coenzyme A, for instance, is derived from the vitamin pantothenic acid; the coenzyme pyridoxal phosphate is derived from vitamin B_6. To cite just one example of how coenzymes function, we consider pyridoxal phosphate. The aldehyde group

can form a covalent complex called a *Schiff base* with an $—NH_2$ group of an amino acid, which facilitates or lowers the activation energy for the breaking of bonds to the carbon of the amino acid. Figure 2-21 shows how pyridoxal phosphate catalyzes the decarboxylation of histidine to form histamine—a potent dilator of small blood vessels. Histamine is released by certain cells in the course of allergenic hypersensitivity.



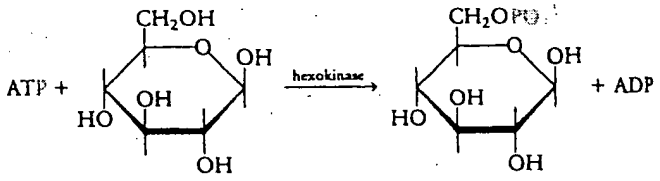
◀ **Figure 2-21** Pyridoxal phosphate, a coenzyme, participates in many reactions involving amino acids. When it is bound to histidine decarboxylase, as in this example, it forms a Schiff base with the α amino group of histidine. The positive charge on the nitrogen of pyridoxal phosphate then attracts the electrons from the carboxylate group of the histidine, via a charge relay system. This weakens the bond between the α carbon of the histidine and the carboxylate group, causing the release of CO_2. Finally, histamine, the reaction product, is hydrolyzed from the pyridoxal complex.

Substrate Binding May Induce a Conformational Change in the Enzyme

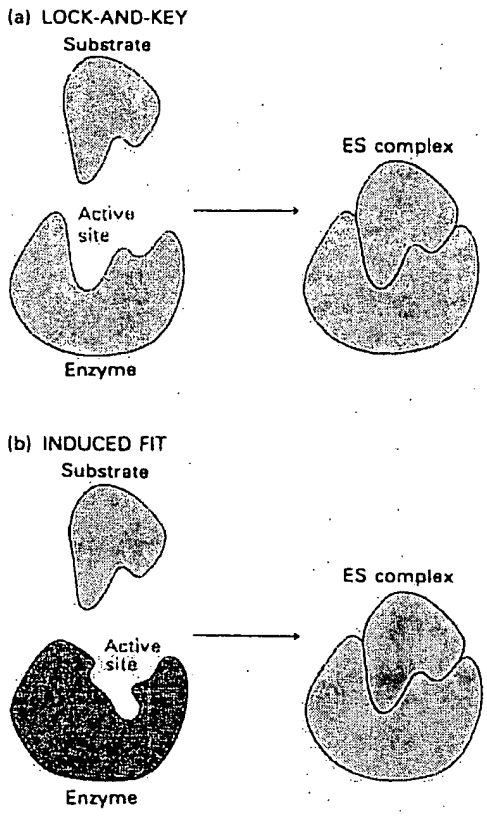
When a substrate binds to an enzyme, molecules of complementary charge or shape, or both, may simply fit together into a complex stabilized by a variety of noncovalent bonds. Such an interaction resembles the fitting of a key into a lock and is said to occur by a *lock-and-key* mechanism (Figure 2-22a).

In some enzymes, the binding of the substrate induces a conformational change in the enzyme that causes the catalytic residues to become positioned correctly. Molecules that attach to the substrate-binding site, or *recognition site*, of the enzyme but that do not induce a conformational change are not substrates of that enzyme. Thus an enzyme differentiates between a substrate and a nonsubstrate in two ways: Does the potential substrate bind to the enzyme? If so, does it induce the correct conformational change? When both criteria are met, the enzyme-substrate complex is said to demonstrate *induced fit* (Figure 2-22b).

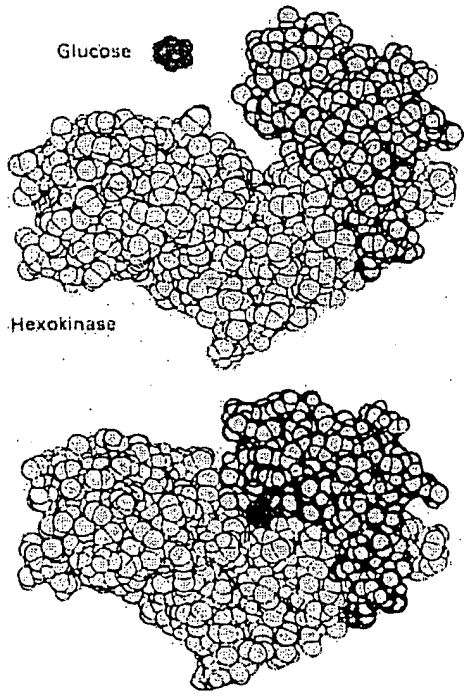
An important example of induced fit is provided by the enzyme hexokinase, which catalyzes the transfer of a phosphate residue from ATP to a specific carbon atom of glucose:



This is the first step in the degradation of glucose by cells. X-ray crystallography has shown that hexokinase consists of two domains. The binding of glucose induces a major conformational change that brings these domains closer together and creates a functional catalytic site (Figure 2-23). Only glucose and closely related molecules can induce this conformational change, ensuring that the enzyme is used to phosphorylate only the correct substrates. Molecules such as glycerol, ribose, and even water may bind to the enzyme at the recognition site but cannot induce the requisite conformational change, so they are not substrates for the enzyme.



▲ **Figure 2-22** Two mechanisms for the interaction of an enzyme and a substrate. (a) In the lock-and-key mechanism, the substrate fits directly into the binding site of the enzyme. (b) If binding occurs by induced fit, the substrate induces a conformational change in the enzyme that appropriately positions the substrate for catalysis.



▲ **Figure 2-23** The conformation of hexokinase changes markedly when it binds the substrate glucose: the two domains of the enzyme come closer together to surround the substrate. Molecules such as the five-carbon sugar ribose can also bind to hexokinase by forming specific hydrogen bonds with groups in the substrate-binding pocket of the enzyme, but only glucose can form all of the bonds that cause the enzyme to change its conformation. *Courtesy of Dr. Thomas A. Steitz.*

The Catalytic Activity of an Enzyme Can Be Characterized by a Few Numbers

Enzymatic specificity is usually quantified by discrimination ratios: a good substrate may be cleaved 10,000 times as fast as a poor substrate. The catalytic power of an enzyme on a given substrate involves two numbers: K_m, which measures the affinity of the enzyme for its substrate, and V_{max}, which measures the maximal velocity of enzymatic catalysis. Equations for K_m and V_{max} are most easily derived by considering the simple reaction

$$S \longrightarrow P$$
$$(\text{substrate} \longrightarrow \text{product})$$

in which the rate of product formation depends on [S], the concentration of the substrate, and on [E], the concentration of the catalytic enzyme. For an enzyme with a single catalytic site, Figure 2-24(a) shows how $d[P]/dt$, the rate of product production, depends on [S] when [E] is kept constant.

At low concentrations of S, the reaction rate is proportional to [S]; as [S] is increased the rate does not increase indefinitely in proportion to [S] but eventually reaches V_{max}, at which it becomes independent of [S]. V_{max} is proportional to [E] and to a catalytic constant k_{cat} that is an intrinsic property of the individual enzyme; halving [E] reduces the rate at all values of [S] by one-half.

When interpreting curves such as those in Figure 2-24, bear in mind that all enzymatically catalyzed reactions include at least three steps: (1) the binding of the substrate (S) to the enzyme (E) to form an enzyme-substrate complex (ES); (2) the conversion of ES to the enzyme-product complex (EP); and (3) the release of the product (P) from EP, to yield free P:

$$E + S \underset{}{\overset{\text{binding}}{\rightleftharpoons}} ES \xrightarrow{\text{catalysis}} EP \xrightarrow{\text{release}} P + E$$

In the simplest case, the release of P is so rapid that we can write

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_{cat}} E + P$$

The reaction rate $d[P]/dt$ is proportional to the concentration of ES and to the catalytic constant k_{cat} for the given enzyme:

$$\frac{d[P]}{dt} = k_{cat}[ES] \quad (1)$$

To calculate [ES], we assume the reaction is in a steady state, so that k_1 [E] [S], the formation rate of [ES], is equal to the rate of its consumption, either by dissociation of uncatalyzed substrate at a rate of k_2 [ES] or by catalysis at a rate of k_{cat} [ES]:

$$k_1[E][S] = (k_2 + k_{cat})[ES] \quad (2)$$

If

$$[E]_{tot} = [E] + [ES] \quad (3)$$

(where $[E]_{tot}$ is the sum of the free and the complexed enzyme, or the total amount of enzyme), then we can combine equations (2) and (3) to obtain

$$[E]_{tot} = [E] + [ES] = \frac{(k_2 + k_{cat})}{k_1[S]}[ES] + [ES]$$

$$= [ES]\left[1 + \left(\frac{k_2 + k_{cat}}{k_1}\right)\left(\frac{1}{[S]}\right)\right]$$

If we define K_m, called the *Michaelis* constant, as

$$\frac{k_2 + k_{cat}}{k_1} \quad (4)$$

then

$$[ES] = \frac{[E]_{tot}}{1 + K_m/[S]}$$

Thus

$$\frac{d[P]}{dt} = k_{cat}[ES] = k_{cat}[E]_{tot}\frac{1}{1 + K_m/[S]}$$

$$= k_{cat}[E]_{tot}\frac{[S]}{[S] + K_m} \quad (5)$$

This equation fits the curves shown in Figure 2-24a. V_{max}, which is equal to $k_{cat}[E]_{tot}$, is the maximal rate of product formation if all recognition sites on the enzyme are filled with substrate. K_m is equivalent to the substrate concentration at which the reaction rate is half-maximal. (If $[S] = K_m$, then from equation (5) we calculate the rate of product formation to be $\frac{1}{2}k_{cat}[E]_{tot} = \frac{1}{2}V_{max}$.) For most enzymes, the slowest step is the catalysis of [ES] to [E] + [P]. In these cases, k_{cat} is much less than k_2, so that $K_m = (k_2 + k_{cat})/k_1 \cong k_2/k_1$ is equal to the equilibrium constant for binding S to E. Thus the parameter K_m describes the affinity of an enzyme for its substrate. The smaller the value of K_m, the more avidly the enzyme can bind the substrate from a dilute solution (Figure 2-24b) and the lower the value of [S] needed to reach half-maximal velocity. The concentrations of the various small molecules in a cell vary widely, as do the K_m values for the different enzymes that act on them. Generally, the intracellular concentration of a substrate is approximately the same as or greater than the K_m value of the enzyme to which it binds.

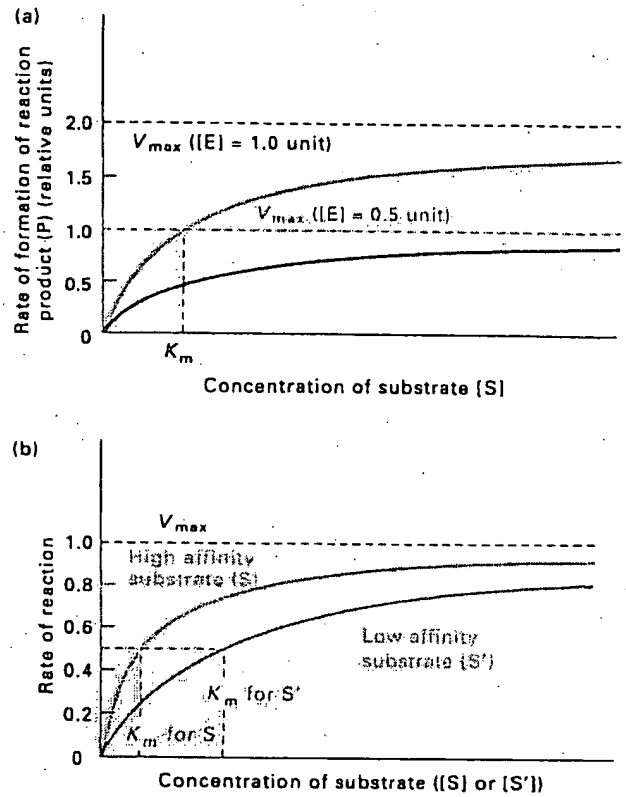
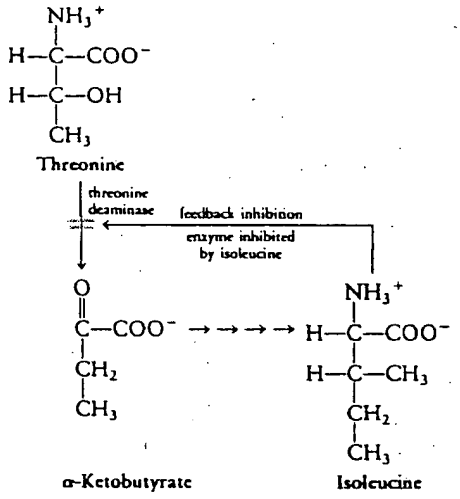


Figure 2-24 (a) The rate of a hypothetical enzymatically catalyzed reaction S → P for two different concentrations of enzyme [E] as a function of the concentration of substrate [S]. The substrate concentration that yields a half-maximal reaction rate is denoted by K_m. Doubling the amount of enzyme causes a proportional increase in the rate of the reaction, so that the maximal velocity V_{max} is doubled. The K_m, however, is unaltered. (b) The rates of reactions catalyzed by an enzyme with a substrate S, for which the enzyme has a high affinity, and with a substrate S', for which the enzyme has a low affinity. The V_{max} value is the same for S and S', but K_m is higher for S'.

The Actions of Most Enzymes Are Regulated

Many reactions in cells do not occur at a constant rate. Instead, the catalytic activity of the enzymes is *regulated* so that the amount of reaction product is just sufficient to meet the needs of the cell.

An Enzyme Can Be Feedback Inhibited in a Reaction Pathway Consider a series of reactions leading to the synthesis of the amino acid isoleucine, which is primarily used by cells as a monomer in the synthesis of proteins. The amount of isoleucine needed depends on the rate of protein synthesis in the cell. The first step in the synthesis of isoleucine is the elimination of an amino group, which converts the amino acid threonine to the compound α-ketobutyrate. Threonine deaminase—the enzyme that catalyzes this reaction—plays a key role in regulating the level of isoleucine. In addition to its substrate-binding sites for threonine, threonine deaminase contains a binding site for isoleucine. When isoleucine is bound there, the enzyme molecule undergoes a conformational change, so that it cannot function as efficiently. Thus isoleucine acts as an *inhibitor* of the reaction for the conversion of threonine. If the isoleucine concentration in the cell is high, the binding of isoleucine to the enzyme temporarily reduces the rate of isoleucine synthesis:



This is an example of *feedback inhibition*, whereby an enzyme that catalyzes one of a series of reactions is inhibited by the ultimate product of the pathway.

In isoleucine synthesis, as in most cases of feedback inhibition, the final product in the reaction pathway inhibits the enzyme that catalyzes the first step that does not also lead to other products. The suppression of enzyme function is not permanent. If the concentration of free isoleucine is lowered, bound isoleucine dissociates from the enzyme, which then reverts to its active conformation. The binding of the inhibitor isoleucine to the enzyme and its subsequent release can be described by the equilibrium-binding constant K_i, which is similar to the constant K_m used for substrate binding:

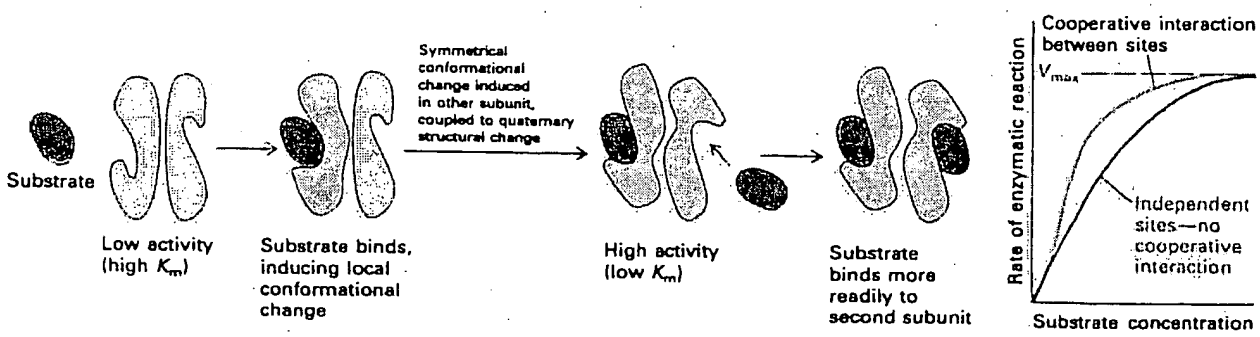
$$[E \cdot Ile]_{inactive} \overset{K_i}{\rightleftharpoons} [Ile] + [E]_{active}$$

$$K_i = \frac{[Ile][E]_{active}}{[E \cdot Ile]_{inactive}}$$

Many Enzymes Have Multiple Binding Sites for Regulatory Molecules Some enzymes have binding sites for small molecules that affect their catalytic activity; a stimulator molecule is called an *activator*. Enzymes may even have multiple sites for recognizing more than one activator or inhibitor. In a sense, enzymes are like microcomputers; they can detect concentrations of a variety of molecules and use that information to vary their own activities. Molecules that bind to enzymes and increase or decrease their activities are called *effectors*. Effectors can modify enzymatic activity because enzymes can assume both active and inactive conformations: activators are positive effectors; inhibitors are negative effectors. Effectors bind at *regulatory sites*, or *allosteric sites* (from the Greek for "another shape"), a term used to emphasize that the regulatory site is an element of the enzyme distinct from the catalytic site and to differentiate this form of regulation from competition between substrates and inhibitors at the catalytic site.

Multimeric Organization Permits Cooperative Interactions among Subunits Many enzymes and some other proteins are multimeric—that is, they contain several copies, or subunits, of one or more distinct polypeptide chains. Some multimeric enzymes contain identical subunits, each of which has a catalytic site and possibly an effector site. In other enzymes, regulatory sites and catalytic sites are located on different subunits, each with a particular structure. On binding an activator, inhibitor, or substrate, a subunit undergoes a conformational change, usually small, that triggers a change in quaternary structure. This quaternary rearrangement favors a similar conformational change in the other subunits, thereby increasing their affinity for the type of ligand initially bound (Figure 2-25). When several subunits interact cooperatively, a given increase or decrease in substrate or effector concentration causes a larger change in the rate of an enzymatic reaction than would occur if the subunits acted independently. Because of such *cooperative interactions*, a small change in the concentration of an effector or substrate can lead to large changes in catalytic activity.

Cooperative interactions among the four subunits in hemoglobin demonstrate clearly the advantages of multimeric organization. The binding of an O_2 molecule to any one of the four chains (each hemoglobin chain binds one O_2) induces a local conformational change in that subunit. This change can in turn induce a large change in quaternary structure. The quaternary change involves a rearrangement of the positions of the two α and two β chains in the tetramer. The local conformational changes that accompany O_2 binding can then occur more readily in the remaining subunits, increasing their affinity for oxygen. The binding of a second O_2 makes the quaternary structural change even more likely. The cooperative



▲ **Figure 2-25** A cooperative interaction between active sites (two identical subunits of a hypothetical enzyme). The binding of a substrate to one subunit of a multimeric enzyme induces a conformational change in the adjacent subunit, which lowers the K_m for the binding of the substrate there. Thus a small change in the substrate concentration can cause a much larger increase in the reaction rate than would occur if there were no cooperative interactions between active sites.

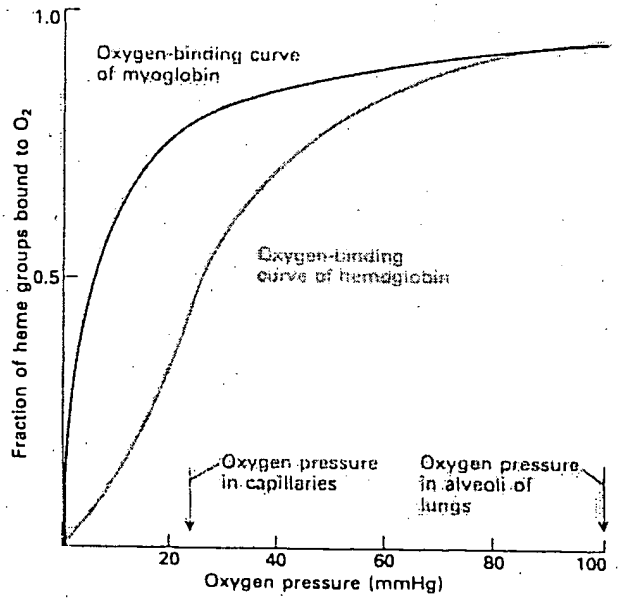
interaction between the chains causes the molecule to take up or lose four O_2 molecules over a much narrower range of oxygen pressures than it would otherwise. As a result, hemoglobin is almost completely oxygenated at the oxygen pressure in the lungs and largely deoxygenated at the oxygen pressure in the tissue capillaries (Figure 2-26).

The contrast between hemoglobin and myoglobin is revealing. Myoglobin is a single-chain oxygen-binding protein found in muscle. The oxygen-binding curve of myoglobin has the characteristics of a simple equilibrium reaction:

$$E + O_2 \overset{K_{O_2}}{\rightleftharpoons} E{-}K_{O_2}$$

Myoglobin has a greater binding affinity for O_2 (a lower K_{O_2}) than hemoglobin at all oxygen pressures. Thus, at the oxygen pressure in capillaries, O_2 moves from hemoglobin into the muscle cells, where it binds to myoglobin, ensuring the efficient transfer of O_2 from blood to tissues.

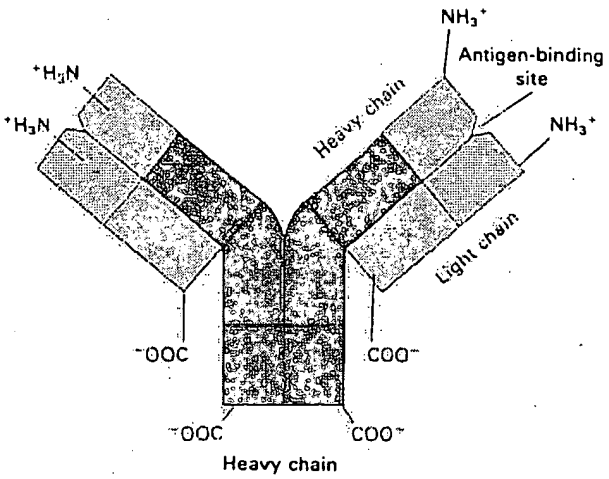
The quaternary-structure rearrangements associated with multimeric organization also provide a way for the effects of activator or inhibitor binding at an allosteric site to be transmitted to a distant catalytic site without large changes in the secondary or tertiary structure of an enzyme, which would be incompatible with the principle that a particular primary structure must adopt a unique folded conformation. Thus, for example, small conformational changes in a domain in response to binding of an effector molecule would produce a quaternary-structure change, which amplifies the conformational signal and allows it to be transmitted robustly to other parts of the enzyme, where it would induce a small conformational change affecting enzymatic activity. Membrane-embedded receptor proteins that must transmit a conformational signal from one side of a membrane to the other are also likely to be multimeric; they transmit the signal by quaternary-structure rearrangement or by an effector-induced shift in the monomer-multimer equilibrium.



◀ **Figure 2-26** The binding of oxygen to hemoglobin depends on cooperative interactions between the four chains. The graph shows the fraction of heme groups in hemoglobin and in myoglobin bound to O_2 as a function of the oxygen pressure. Note that the binding activity of hemoglobin increases sharply over a narrow range of oxygen pressures (20–40 mmHg). Hemoglobin is saturated with O_2 in the lungs, but it releases much of its bound O_2 at the low oxygen pressure in the tissue capillaries. At any oxygen pressure, myoglobin has a higher affinity for O_2 than hemoglobin does. As myoglobin is a principal muscle protein, this property allows oxygen to be transferred from blood to muscle.

Enzymes Are Regulated in Many Ways The activities of enzymes are extensively regulated so that the numerous enzymes in a cell work together harmoniously. All metabolic pathways are closely controlled at all times. Synthetic reactions occur when the products of these reactions are needed; degradative reactions occur when molecules must be broken down. Kinetic controls affecting the activities of key enzymes determine which pathways are going to be used and the rates at which they will function.

Regulation of cellular processes involves more than simply turning enzymes on and off, however. Some regulation is accomplished through *compartmentation*. Many enzymes are localized in specific compartments of the cell, such as the mitochondria or lysosomes, thereby restricting the substrates, effectors, and other enzymes with which an enzyme can interact. In addition, compartmentation permits reactions that might otherwise compete with one another in the same solution to occur simultaneously in different parts of a cell. Cellular processes are also regulated through the control of the rates of enzyme synthesis and destruction.



△ **Figure 2-27** The structure of an antibody molecule illustrated in an immunoglobulin (IgG) made of four polypeptide chains: two identical heavy chains (blue) and two identical light chains (orange). Each antigen-binding site is formed by the N-terminal segments of a heavy and a light chain. The N-termini are highly variable in sequence, giving rise to the wide range of antibody specificity.

Antibodies

Enzymes are not the only proteins that bind tightly and specifically to smaller compounds. The insulin receptor on the surface of a liver cell, for example, can bind to insulin so tightly that the receptors on a cell are half-saturated when the insulin concentration is only 10^{-9} *M*. This protein does not bind to most other compounds present in blood; it mediates the specific actions of insulin on liver cells. A molecule other than an enzyme substrate that can bind specifically to a macromolecule is often called a *ligand* of that macromolecule.

The capacity of proteins to distinguish among different molecules is developed even more highly in blood proteins called *antibodies*, or *immunoglobulins*, than in enzymes. Animals produce antibodies in response to the invasion of an infectious agent, such as a bacterium or a virus. Antibodies will be discussed at length in Chapter 25. We introduce them here because they will appear as critical reagents in the discussions of many intervening chapters.

The recognition site of an antibody can bind tightly to very specific sites—generally on proteins or carbohydrates—on the surface of the infectious agent. Experimentally, animals produce antibodies in response to the injection of almost any foreign polymer; such antibodies bind specifically and tightly to the invading substance but, like enzymes, do not bind to dissimilar molecules. The antibody acts as a signal for the elimination of infectious agents. When it binds to a bacterium, virus, or virus-infected cell, certain white blood cells (leucocytes) recognize the invading body as foreign and respond by destroying it. The specificity of antibodies is exquisite: they can distinguish between proteins that differ by only a single amino acid and between the cells of different individual members of the same species.

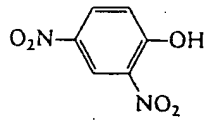
All vertebrates can produce a large variety of antibodies, including ones that bind to chemically synthesized molecules. Exposure to an antibody-producing agent, called an *antigen*, causes an organism to make a large quantity of different antibody proteins, each of which may bind to a slightly different region of the antigen. For a given antigen, these constellations of antibodies may differ from one member of a species to another.

Antibodies are formed from two types of polypeptides: heavy chains, each of which is folded into four domains, and light chains, each of which is folded into two domains (Figure 2-27). The N-terminal domains of both heavy and light chains are highly variable in sequence, giving rise to the specific binding characteristics of antibodies.

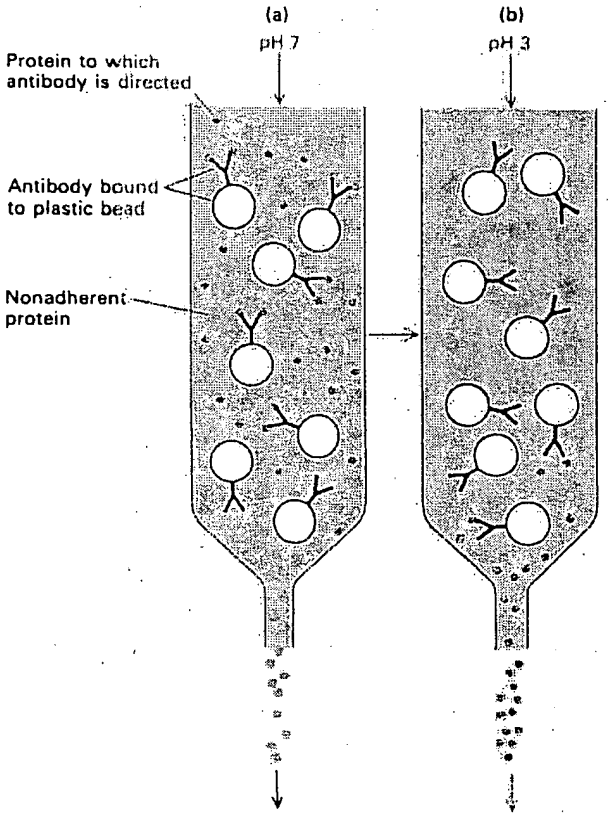
Antibodies Can Distinguish among Closely Similar Molecules

The sequence of bovine insulin is identical to that of human insulin, except at three amino acids. Yet when bovine insulin is injected into people, some individuals respond by synthesizing antibodies that specifically recognize the specific amino acids in the bovine molecule, even though human beings generally do not produce anti-

bodies that recognize their own insulin. Injecting mouse albumin—the major serum protein—into mice does not elicit the production of antialbumin antibodies. However, if a small molecule, such as 2,4-dinitrophenol (DNP)



is coupled to the albumin, mice do produce antibodies that bind specifically to the modified region of the protein—in this case, to the dinitrophenyl group. A small group capable of eliciting antibody production is called a *hapten*. The anti-DNP/albumin antibody will not bind to albumin that is not complexed with DNP or that is modified by other haptens (even phenyl groups with different substituents).



▲ **Figure 2-28** The purification of a protein from a mixture by affinity chromatography. (a) In step 1, the mixture is filtered through a column containing antibody molecules that are specific for the desired protein. Only that protein binds to the antibody matrix; any other proteins in the mixture are eluted. (b) In step 2, a solution such as acetic acid is added to disrupt the antigen-antibody complex, so that a pure protein is eluted from the column.

Antibodies Are Valuable Tools for Identifying and Purifying Proteins

Because they bind so selectively to proteins, antibodies can be used experimentally to isolate one protein from a complex mixture. In one technique, *affinity chromatography*, a pure antibody is chemically coupled to tiny plastic beads, which are then placed in a small column. When a protein solution is applied, only the protein to which the antibody is directed adheres to the column; all nonadherent proteins pass through the column unimpeded (Figure 2-28a). The adherent protein can then be eluted by adding a solution that disrupts the binding between the protein and the antibody (Figure 2-28b). Similarly, antibodies can be used to detect specific proteins in cells or other biological materials.

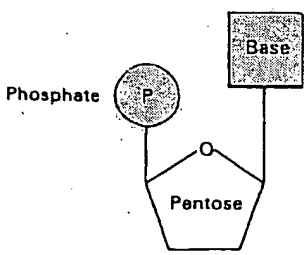
Nucleic Acids

Cells receive instructions about which proteins to synthesize and in what quantities from *nucleic acids*—the molecules that store and transmit information in cells. As in many systems of communication, this information is processed in the form of a code. The translation of this code is described in Chapter 3. Here, we examine the chemical structures of the molecules that store the encoded information.

Nucleic Acids Are Linear Polymers of Nucleotides Connected by Phosphodiester Bonds

Cells have two closely related information-carrying molecules: deoxyribonucleic acid (DNA) and ribonucleic acid (RNA). Like proteins, DNA and RNA are linear polymers. However, the number of monomers in a nucleic acid is generally much greater than the number of amino acids in a protein. Cellular RNAs range in length from tens to thousands of units. The number of units in a DNA molecule can be in the millions.

DNA and RNA each consist of only four different monomers, called *nucleotides*. A nucleotide has three parts: a phosphate group, a *pentose* (a five-carbon sugar molecule), and an organic *base* (Figure 2-29). In RNA,



◀ **Figure 2-29** A schematic diagram of the structure of a nucleotide.